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Intra- and Interspecies Effects of Outer Membrane Vesicles from *Stenotrophomonas maltophilia* on β-Lactam Resistance

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The treatment of *Stenotrophomonas maltophilia* infection with β-lactam antibiotics leads to increased release of outer membrane vesicles (OMVs), which are packed with two chromosomally encoded β-lactamases. Here, we show that these β-lactamase–packed OMVs are capable of establishing extracellular β-lactam degradation. We also show that they dramatically increase the apparent MICs of imipenem and ticarcillin for the cohabituating species *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*.

The multidrug-resistant bacterium *Stenotrophomonas maltophilia* exploits a variety of mechanisms to resist antibiotic threats, such as the active extrusion of antibiotics by efflux pumps, alteration of cell membrane permeability, shielding by growing as a biofilm, and direct enzymatic inactivation of the antibiotic compounds (1). β-Lactam antibiotics, such as imipenem (IPM), amoxicillin (AMX), and ticarcillin (TIC), are used frequently to treat Gram-negative bacterial infections. Unfortunately, these compounds are often ineffective for *S. maltophilia* infections due to the presence of two chromosomal β-lactamase genes encoding the L1 metallo-β-lactamase and the L2 serine-β-lactamase, the expression of which immediately increases after exposure (2). In a previous study, we revealed a significant increase in the release of outer membrane vesicles (OMVs) by the clinical *S. maltophilia* strain 44/98 (LMG 26824) after exposure to the broad-spectrum carbapenem IPM, and we showed that these vesicles are packed with L1 and L2 β-lactamases (3).

In this study, OMVs from penicillin G (PEN)-stimulated cultures (1 mg/ml, sublethal concentration) were used. The culture supernatant was filtered through a syringe-driven 0.22-μm-pore-size polyethersulfone membrane filter unit, and the OMVs were pelleted by ultracentrifugation at 100,000 × *g* for 1 h. The OMVs were quantified with fluorescent single-particle tracking with the membrane-specific fluorescent PKH67 label (4, 5). The measured increase in levels of secreted membranous particles after exposure to PEN was comparable to that measured after exposure to IPM (17.3-fold increase). PEN-induced OMVs were also subjected to a two-dimensional liquid chromatography–mass spectrometry proteomics study to identify the protein cargo, as previously described (3). The two β-lactamases were again found to be included in the OMVs.

The β-lactamase activity on intact OMVs was examined using a nitrocefin β-lactamase assay. OMVs (isolated from 25 ml PEN-stimulated culture, dissolved in 1 ml phosphate-buffered saline [PBS]) were mixed with 50 μl of a 0.5-mg/ml nitrocefin solution, and the optical density at 490 nm (OD_{490}) was measured at different time points. The results show rapid nitrocefin hydrolysis by the β-lactamase–packed OMVs (Fig. 1A). The rate of hydrolysis, derived from the linear part of the curve (5 to 15 min), was calculated as 0.571 μg/min. To assess the contribution of the L1 metallo-β-lactamase, the same assay was performed after incubating the OMVs with the zinc-chelating agent EDTA (6). The initial rate of hydrolysis was then 0.262 μg/min, approximately half of the rate observed without EDTA. This demonstrates the OMV-associated

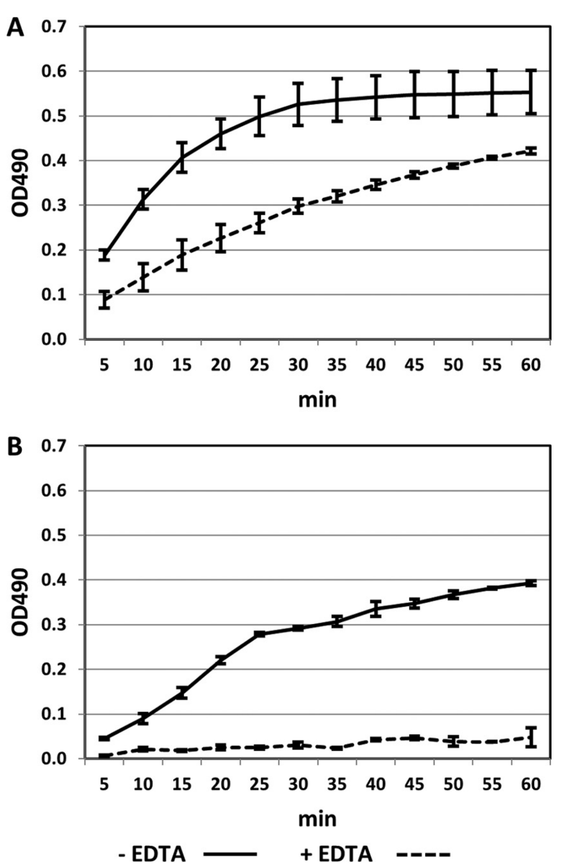

FIG 1 UV visible spectroscopy analysis of nitrocefin hydrolysis (OD_{490}) at different time points during incubation with intact PEN-induced OMVs (A) and crude PEN-stimulated culture lysate (B), with and without the addition of EDTA. Error bars show the standard deviation (SD).

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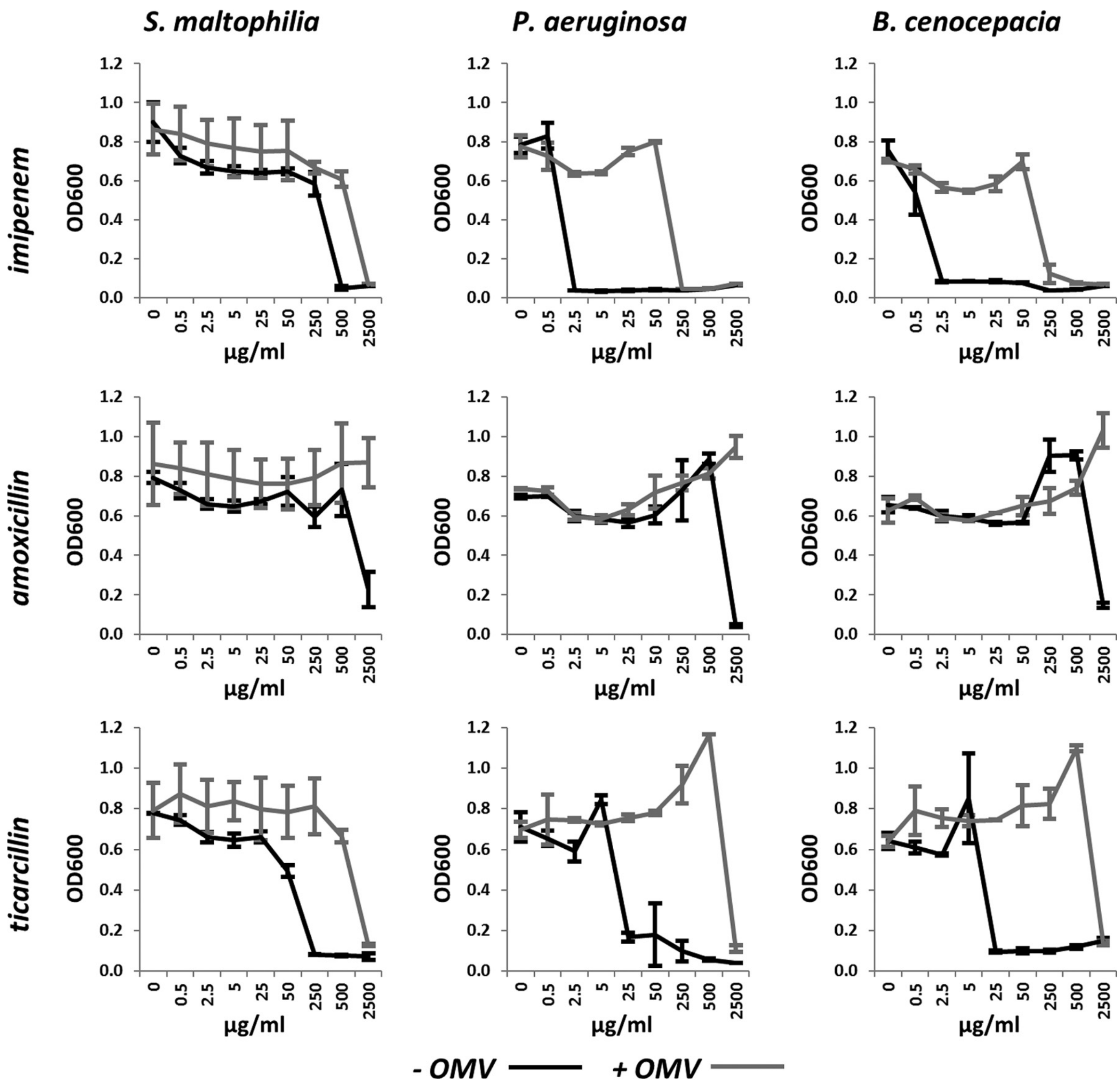

FIG 2 OD_{600} of *S. maltophilia*, *P. aeruginosa*, and *B. cenocepacia* cultures exposed to different concentrations of IPM, AMX, and TIC in the absence and in the presence of PEN-induced OMVs. Error bars show the SD.

activity of both β-lactamases. The nitrocefin assay was also performed on crude culture lysate. Cells (isolated from 500 μl PEN-stimulated culture, dissolved in 500 μl PBS) were lysed by bead milling, and the clear lysate was transferred to a new Eppendorf tube. The volume was adjusted to 1 ml with PBS for the nitrocefin assay. It was remarkable that the crude culture lysate showed almost complete activity loss after the addition of EDTA (Fig. 1B), which points to a dominant role for L1 in cellular β-lactam resistance, as previously reported (7). However, the OMVs seemed to have had an equal activity distribution between L1 and L2.

As *S. maltophilia* is often part of polymicrobial communities, we investigated whether OMVs from *S. maltophilia* influence the tolerance of *Pseudomonas aeruginosa*, *Burkholderia cenocepacia*, and itself to the β-lactam antibiotics. Cultures from *S. maltophilia* strain 44/98, *P. aeruginosa* strain PAO1 (LMG 24986), and a *B. cenocepacia* type strain (LMG 16656; 100 μl) were grown in 96-well plates with different concentrations of the antibiotics IPM, AMX, and TIC (0, 0.5, 2.5, 5, 25, 50, 250, 500, and 2,500 μg/ml), with or without OMVs derived from a PEN-stimulated *S. maltophilia* culture (isolated from a 2.5-ml culture, dissolved in 100 μl fresh Luria Bertani medium). *S. maltophilia* showed high resistance toward the three β-lactam antibiotics, with growth inhibition at 500, 2,500, and 250 μg/ml of IPM, AMX, and TIC, respectively (Fig. 2, left column). When isolated OMVs (containing β-lactamases) were added, the MICs increased to 2,500 μg/ml for IPM and TIC and were even higher for AMX (>2,500 μg/ml). The effects of the antibiotics and the OMVs on *P. aeruginosa* and *B. cenocepacia* were very much alike (Fig. 2, middle and right columns). Both species were as resistant to AMX as *S. maltophilia*, and the addition of the OMVs also led to an increased MIC (>2,500 μg/ml). *P. aeruginosa* and *B. cenocepacia* are naturally less resistant to IPM and TIC, but the presence of *S. maltophilia* OMVs drastically increased the antibiotic tolerance of these species. A 100-fold increase in MICs was observed, from 2.5 to 250 μg/ml and 25 to 2,500 μg/ml for IPM and TIC, respectively.

We have shown here that *S. maltophilia* β-lactamase–packed

OMVs indeed exhibit β-lactamase activity. Moreover, the OMVs provide the enzymes shelter against proteases, keeping them stable and active for longer periods (8). The extracellular β-lactamase activity associated with the OMVs can thereby also affect other species, possible cohabitants in polymicrobial communities. It was shown previously that *S. maltophilia* often lives together with the species *P. aeruginosa* and *B. cenocepacia*, especially in the lungs of cystic fibrosis patients, where these species are found in polymicrobial biofilm communities (9). In conclusion, the exposure of *S. maltophilia* to β-lactam antibiotics leads to the secretion of β-lactamase–packed OMVs, which in turn can protect not only other *S. maltophilia* cells but also *P. aeruginosa* and *B. cenocepacia* against β-lactam antibiotics. Although it is not clear whether *S. maltophilia* can be considered a true cystic fibrosis pathogen (10), its ability to secrete OMVs after antibiotic stress can influence the susceptibility of the pathogens to antibiotic treatment.

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REFERENCES

1. **Abbott IJ, Slavin MA, Turnidge JD, Thursky KA, Worth LJ.** 2011. *Stenotrophomonas maltophilia*: emerging disease patterns and challenges for treatment. Expert Rev Anti Infect Ther **9:**471–488. http://dx.doi.org/10.1586/eri.11.24.
2. **Brooke JS.** 2012. *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. Clin Microbiol Rev **25:**2–41. http://dx.doi.org/10.1128/CMR.00019-11.
3. **Devos S, Van Oudenhove L, Stremersch S, Van Putte W, De Rycke R, Van Driessche G, Vitse J, Raemdonck K, Devreese B.** 2015. The effect of imipenem and diffusible signaling factors on the secretion of outer membrane vesicles and associated Ax21 proteins in *Stenotrophomonas maltophilia*. Front Microbiol **6:**298. http://dx.doi.org/10.3389/fmicb.2015.00298.
4. **Braeckmans K, Buyens K, Bouquet W, Vervaet C, Joye P, De Vos F, Plawinski L, Doeuvre L, Angles-Cano E, Sanders NN, Demeester J, De Smedt SC.** 2010. Sizing nanomatter in biological fluids by fluorescence single particle tracking. Nano Lett **10:**4435–4442. http://dx.doi.org/10.1021/nl103264u.
5. **Roding M, Deschout H, Braeckmans K, Rudemo M.** 2011. Measuring absolute number concentrations of nanoparticles using single-particle tracking. Phys Rev E Stat Nonlin Soft Matter Phys **84:**031920. http://dx.doi.org/10.1103/PhysRevE.84.031920.
6. **Hu RM, Chiang KH, Lin CW, Yang TC.** 2008. Modified nitrocefin-EDTA method to differentially quantify the induced L1 and L2 β-lactamases in *Stenotrophomonas maltophilia*. Lett Appl Microbiol **47:**457–461. http://dx.doi.org/10.1111/j.1472-765X.2008.02438.x.
7. **Van Oudenhove L, De Vriendt K, Van Beeumen J, Mercuri PS, Devreese B.** 2012. Differential proteomic analysis of the response of *Stenotrophomonas maltophilia* to imipenem. Appl Microbiol Biotechnol **95:**717–733. http://dx.doi.org/10.1007/s00253-012-4167-0.
8. **Aldick T, Bielaszewska M, Uhlin BE, Humpf HU, Wai SN, Karch H.** 2009. Vesicular stabilization and activity augmentation of enterohaemorrhagic *Escherichia coli* haemolysin. Mol Microbiol **71:**1496–1508. http://dx.doi.org/10.1111/j.1365-2958.2009.06618.x.
9. **Raidt L, Idelevich EA, Dübbers A, Küster P, Drevinek P, Peters G, Kahl BC.** 2015. Increased prevalence and resistance of important pathogens recovered from respiratory specimens of cystic fibrosis patients during a decade. Pediatr Infect Dis J **34:**700–705. http://dx.doi.org/10.1097/INF.0000000000000714.
10. **Hansen CR.** 2012. *Stenotrophomonas maltophilia*: to be or not to be a cystic fibrosis pathogen. Curr Opin Pulm Med **18:**628–631. http://dx.doi.org/10.1097/MCP.0b013e328358d4f8.